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14. ABSTRACT:
Learning disabilities severely deteriorate the life of many NF1 patients. However, the pathogenic process for NF1-associated learning disabilities has not been fully understood and an effective therapy is not available. This study was proposed to identify genes that are dysregulated in the hippocampus of the Nf1+/- mouse model by DNA microarray analysis. Characterization of these NF1-affected genes will dramatically improve our understanding of the molecular pathogenesis underlying NF1-associated learning deficits. During the third year of the project, we have focused on determining the biological functions of NF1-affected genes. In addition, we also characterized the effect of lovastatin treatment on genomic expression of the NF1 hippocampus.

15. SUBJECT TERMS
NF1, hippocampus, learning disabilities, LTP, microarray, gene

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Introduction

Learning disabilities severely deteriorate the life of many NF1 children by limiting their academic achievement, higher education and career choice (1). However, the pathogenic process for NF1-associated learning disabilities has not been fully understood and an effective therapy is not available. Drs Silva's and Zhong's laboratories have demonstrated that *Nf1* mutations lead to the development of learning deficits in mouse and *Drosophila*, respectively (2-4). Their work suggests that *Nf1* mutations cause learning deficits by disturbing the Ras/MAPK and/or cAMP signaling. Despite these significant progresses, NF1-affected downstream genes that directly contribute to deficits in synaptic plasticity and learning are largely unknown. We proposed to identify genes that are dysregulated in the hippocampus of the $Nf1^{+/-}$ mouse model. Characterization of these NF1-affected genes will dramatically improve our understanding of the molecular pathogenesis underlying NF1-associated learning deficits.

Body

During the third year of this project, we focused on following research activities outlined in the Revised Statement of Work approved last year:

1. Completed microarray data confirmation and bioinformatics analysis (Task 1). In previous year, we finished DNA microarray experiments with purified RNA from the hippocampus of wild-type and NF1 mice, using Affymetrix GeneChips (MG-430). In this year, we carried out work in the following two aspects to complete Task 1. (1). Data confirmation. We performed real-time RT-PCR experiments to confirm our microarray results. The PCR analysis on a group of genes indicated that the expression changes revealed by our DNA microarray analysis are very reliable. (2). Bioinformatic analysis of NF1-affected genes identified by the DNA microarray analysis. We performed extensive bioinofrmatic analysis on NF1-affected genes, and found that many of them are involved in the regulation of synaptic transmission, synaptic plasticity and memory formation. For example, αCaMKII and CREB1 are disturbed in the NF1 hippocampus. Our findings provide a possible molecular etiologic mechanism for impaired synaptic plasticity and memory of NF1 mice, and probably NF1 patients.
2. Completed DNA microarray analysis on the NF1 hippocampus from mice after lovastatin treatment. Last year, our recommendation to change the Task 2 in the original proposal was approved. In the revised Task 2, we proposed to determine the genomic expression pattern in the NF1 hippocampus after lovastatin treatment, which, as shown by D. Silva's lab, is sufficient to rescue memory deficits of NF1 mice. In this year, we have completed the DNA microarray analysis to compare the genomic expression patterns in the hippocampus of lovastatin-treated NF1 and sham-treated NF1 mice. We found that lovastatin treatment changed the expression of many genes in the NF1 hippocampus. A substantial portion of them are NF1-affected genes. Importantly, we observed

reversal effects of lovastatin treatments on the NF1-altered expression of many genes. It would be interesting for future research to determine the role of lovastatin-“corrected” NF1-affected genes in the pathogenesis of NF1 memory deficits.

Key Research Accomplishments

1. Confirmed DNA microarray data and completed bioinformatics analysis on NF1-affected genes.
2. Performed DNA microarray analysis to determine the effect of lovastatin treatment on NF1-affected genes.

Reportable Outcomes

We have submitted a manuscript from this research to report our results outlined above (see attached manuscript). By performing the described research work, two trainees (one postdoctoral fellow and one young research technician) gained experiences in NF1 research.

Conclusions

In summary, we have successfully completed the DNA microarray analysis on the NF1 hippocampus. This analysis has led to the identification of specific NF1-affected genes that are involved in the regulation of synaptic structures and functions. These progresses will facilitate the investigation of the molecular pathways that are disturbed in the NF1 hippocampus and underlie NF1-associated learning disabilities. We also determined the effect of lovastatin on gene expression in the NF1 hippocampus, and identified a group of NF1-affected genes whose abnormal expression in the NF1 hippocampus can be reserved by lovastatin. These findings provide new insights into the molecular abnormalities that probably underlie the pathogenesis of NF1 memory deficits.

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Aberrant expression of synaptic plasticity-related genes in the NF1$^{+/-}$ mouse hippocampus

Running Title: Genomic analysis of the NF1$^{+/-}$ hippocampus

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ABSTRACT

Neurofibromatosis 1 is (NF1) is a common single-gene disorder that causes learning impairments in patients. Neurofibromin encoded by the NF1 causal gene regulates Ras/MAPK and cAMP signaling pathways. These signaling pathways play critical roles in controlling gene transcription during synaptic plasticity and memory formation. We hypothesized that NF1 mutations disturb the expression of genes important for memory formation. To test this hypothesis, we performed DNA microarray analysis on the hippocampus of $NF1^{+/-}$ mice, the mouse model for NF1 learning disabilities. Our results indicated that genes involved in a wide spectrum of biological processes are dysregulated in the $NF1^{+/-}$ hippocampus. Many of the NF1-affected genes play critical roles in synaptic plasticity, such as Rabs, synaptotagmins, NMDAR1, CaMKII and CREB1. We also determined the effect of lovastatin treatment on genomic expression patterns of the $NF1^{+/-}$ hippocampus. We found that lovastatin altered the expression of a large number of genes, including those disturbed by NF1 mutations. Our results reveal a genomic overview of the molecular abnormalities in the $NF1^{+/-}$ hippocampus, and should be useful for further identifying the novel molecular pathways that cause NF1 learning deficits.

Key Words: neurofibromatosis 1, NF1, hippocampus, DNA microarray, synapse, synaptic plasticity, memory, learning disabilities

INTRODUCTION

Neurofibromatosis 1 (NF1) is a prevailing autosomal dominant genetic disorder that approximately occurs 1 in 3500, and is caused by mutations in a single gene, *Nf1* (Eliason, 1988; Cichowski and Jacks, 2001). In addition to a variety of physical manifestations including benign and malignant brain tumors, NF1 patients may also experience cognitive difficulties that are often considered as hallmarks of this disease (Hofman et al., 1994; North, 2000; Cichowski and Jacks, 2001). Among cognitive impairments associated with NF1, learning disabilities (LD) manifested in 30-65% of NF1 children are especially problematic (Eliason, 1988; North, 1993; North et al., 1995; Ferner et al., 1996; Kayl and Moore, 2000).

Heterozygous mice with a *Nf1* null mutation ($Nf1^{+/-}$) develop multiple behavioral phenotypes that bear striking similarities to learning disabilities seen in NF1 patients (Silva et al., 1997; Costa and Silva, 2003). For example, as in NF1 patients, $Nf1^{+/-}$ mice have spatial learning deficits, as suggested by the Morris Water maze test (Silva et al., 1997), a learning task that is sensitive to hippocampal lesions. Importantly, similar to NF1 patients, the learning deficits of $Nf1^{+/-}$ mice can also be compensated by extended training (Silva et al., 1997). However, simple associative learning is not affected in both NF1 patients and $Nf1^{+/-}$ mice (Silva et al., 1997). Similar to NF1 patients, some $Nf1^{+/-}$ mice do not experience learning impairments (Silva et al., 1997). Interestingly, the homozygous knockout mice which carry $Nf1^{23a-/-}$ mutant genes lacking exon 23a also develop learning deficits even though they are developmentally normal and are without an increased tumor predisposition (Costa et al., 2001). This observation indicates that *NF1*-associated learning impairments are specific phenotypes that can be dissociated from developmental and other physical defects. A variety of studies have suggested that long-term

potentiation (LTP) is an important cellular substrate for learning and memory (Grimwood et al., 2001). Consistent with this notion, the hippocampus from *Nf1*$^{+/-}$ mice also displays deficits in LTP expression (Costa et al., 2002).

The neurofibromin protein encoded by the *Nf1* gene is a tumor suppressor related to Ras GTPase-activating proteins (GAP) (Cichowski and Jacks, 2001; Zhu and Parada, 2001a; Weeber and Sweatt, 2002). As a Ras GAP-related protein, neurofibromin functions as a negative regulator of Ras function, by accelerating the hydrolysis of GTP to GDP that leads to inactivation of Ras (Cichowski and Jacks, 2001; Zhu and Parada, 2001a; Weeber and Sweatt, 2002). Previous studies indicated that the abnormal up-regulation of the Ras activity after loss-of-function of neurofibromin is a crucial step leading to learning impairments in *Nf1*$^{+/-}$ mice (Costa et al., 2002). Down-regulation of Ras activities in *Nf1*$^{+/-}$ mice by genetic and pharmacological approaches rescued learning deficits and LTP deficits (Costa et al., 2002). *Drosophila* neurofibromin not only is a Ras-GAP (Williams et al., 2001) but also regulates the cAMP signaling (Guo et al., 1997; The et al., 1997; Guo et al., 2000; Tong et al., 2002). Mutations of *Drosophila Nf1* likely impair learning by inhibiting the cAMP/PKA signaling pathway (Guo et al., 2000). A role of mammalian neurofibromin in regulating adenylyl cyclase has also been reported (Tong et al., 2002). As both Ras/MAPK and cAMP signaling cascades play important roles in regulation of gene expression during synaptic plasticity and memory formation, their disturbance likely dysregulates gene expression in specific brain regions such as the hippocampal formation, where neurofibromin is highly expressed (Nordlund et al., 1995; Zhu and Parada, 2001b). However, NF1-affected genes that contribute to the pathogenesis of learning deficits have not been identified.

We have used DNA microarrays to identify genes that alter their expression in the hippocampus *Nf1*$^{+/-}$ mice. A comprehensive pool of NF1-affected hippocampal genes that function in a wide spectrum of biological pathways is identified. Many of them are known to involve in synaptic plasticity and memory formation. In addition, we also characterized genes that respond to lovastatin treatment. These findings provide a genomic overview of the molecular pathogenic abnormalities that are likely relevant to NF1 learning deficits.

RESULTS

NF1$^{+/-}$ mice develop reversible deficits in consolidation but not in encoding of fear memories.

We first investigated the learning performance of NF1$^{+/-}$ mice on a contextual discrimination task (Frankland et al., 1998). In this hippocampal-dependent learning task, mice were trained in two chambers; one of them was associated with electric shocks (shock chamber) and another was not (control chamber). The expression of freezing behaviors was determined to measure fear memories (Fig. 1). On the training day, NF1$^{+/-}$ and wild-type mice were indistinguishable in both chambers (Fig. 1A). One day after training, both NF1$^{+/-}$ and wild-type mice showed clear increases of freezing behaviors in the shock chamber but not in the control chamber, indicating formation of the contextual-shock association. At this stage, the percentage of freezing developed in NF1$^{+/-}$ mice was not significantly different from that of wild-type mice ($p>0.05$) (Fig. 1B). This observation indicates that the NF1$^{+/-}$ mice are normal during the encoding phase of fear memories. Consistent with this notion, previous work also suggested that

$NF1^{+/-}$ mice are not impaired during the acquisition phase in the Morris Water Maze task (Silva et al., 1997). On the other hand, memory tests on day 7 showed that the percentage of freezing of $NF1^{+/-}$ mice was significantly lower than that of wild-type mice (Fig. 1C). These observations suggest that $NF1^{+/-}$ mice are impaired in memory consolidation between day 2 and 7 after training. To test whether this memory deficiency of $NF1^{+/-}$ mice can be compensated by more training, we added one more session of contextual-shock pairing in the shock chamber at day 1 after the initial training. Memory tests at day 7 showed that, after reinforced training at day 1, $NF1^{+/-}$ and wild-type mice developed similar amounts of freezing (Fig. 1D). This result indicates that the deficiency in contextual fear memory of $NF1^{+/-}$ mice can be compensated by excess training. This observation supports the notion from previous studies in the Morris Water Maze task (Silva et al., 1997).

DNA microarray analysis of the $NF1^{+/-}$ hippocampus.

Results described above and previously (Silva et al., 1997) strongly suggest a specific impairment of $NF1^{+/-}$ mice in consolidation of hippocampal-dependent memory. To obtain insight into the molecular basis of NF1-associated memory impairments, we sought to determine the genomic expression of the $NF1^{+/-}$ hippocampus. We performed DNA microarray analysis to identify genes that were aberrantly expressed in the $NF1^{+/-}$ hippocampus from male mice at 2 month of age, using Affymetrix GeneChip (MG430v2) that contains over 39000 probe sets. Four independent array replicates with RNAs from different mice were included. Visualization of microarray data in a distribution scatter plot revealed that the expression levels of some genes were shifted in the $NF1^{+/-}$ hippocampus (Fig. 2). T-tests with Cyber-T (Hung et al., 2002) (http://visitor.us.uci.edu/genex/cybert/) identified 6418 probes that were significantly changed in

the $NF1^{+/-}$ hippocampus ($p<0.05$) (Supplemental Table 1). We performed real-time RT-PCR analysis to confirm the expression changes of a group of genes (Fig. 3).

Diverse molecular and cellular processes are disturbed in the $NF1^{+/-}$ hippocampus.

To obtain insights into the biological processes disturbed in the $NF1^{+/-}$ hippocampus, we performed gene ontology (GO) analysis of NF1-affected genes (http://www.affymetrix.com/analysis/index.affx; http://www.genmapp.org). The results indicate that genes disturbed in the $NF1^{+/-}$ hippocampus are associated with a variety of molecular and cellular processes, such as cell-cell communication, signal transduction, cellular transport, transcription and cytoskeletons (Table 1). These observations indicate that a wide range of biological processes are disturbed in the $NF1^{+/-}$ hippocampus. Interestingly, previous DNA microarray studies have suggested that spatial learning in the Morris water maze regulates genes in many of these processes, including cell signaling, cell-cell interaction, transcriptional and translational regulation and regulation of cytoskeletons (Cavallaro et al., 2002). Thus, it is likely that there is a globe disturbance of genes in cellular and molecular processes underlying memory formation in the hippocampus of $NF1^{+/-}$ mice.

Dysregulation of synapse-related genes in the $NF1^{+/-}$ hippocampus.

Synapses are the structural units for information storage. We next sought to determine whether synapse-related genes are disturbed in the $NF1^{+/-}$ hippocampus. By searching the PubMed database on the NF1-affected genes ($p<0.05$), we found that 213 were synapse-related (Fig. 4A). 103 of them were aberrantly downregulated and 110 up-regulated. Hierarchical clustering of these synapse-related genes showed that many of the synapse-related genes were

consistently dysregulated in the NF1$^{+/-}$ hippocampus from different animals (Fig. 4). Dysregulation of these genes may be a reliable biomarker of the NF1$^{+/-}$ hippocampus.

Of the synapse-related genes dysregulated in the NF1$^{+/-}$ hippocampus, many of them are involved in neurotransmitter vesicle trafficking/recycling (Table 2). These include Rab and synaptotagmin proteins, such as Rab1, Rab2, Rab3A, and synaptotagmin1 (Table 2; Supplemental Table 1). In addition, the expression of several synaptic receptor genes, including NMDA receptor 1, AMPA receptor 4 and metabotropic glutamate receptor 5 (mGluR5), was altered (Table 2; Supplemental Table 1). These observations indicate that genes critical for neurotransmission are disturbed in the NF1$^{+/-}$ hippocampus. Furthermore, a number of genes encoding synaptic structural proteins, such as neurexin1, integrin β6, integrin β7 and Ncam1, were disturbed (Table 2; Supplemental Table 1). We also observed that genes encoding CaMKII and other important synaptic signaling proteins were dysregulated in the NF1$^{+/-}$ hippocampus (Table 2). The altered expression of the genes involved in the regulation of neurotransmission, structures and signaling of the synapse potentially contributes to the observed impairments of synaptic function and plasticity (Costa et al., 2002).

Disturbance of LTP-regulated genes in the NF1$^{+/-}$ hippocampus.

Long-term potentiation (LTP) is widely considered as a critical cellular mechanism underlying memory formation. We hypothesized that specific LTP-related genes are disturbed in the NF1$^{+/-}$ hippocampus. To test this idea, we compared the activity-regulated genes (ARGs) that changed their expression after LTP induction (Park et al., 2006) with those that were disturbed in the NF1$^{+/-}$ hippocampus (Supplemental Table 1). We found that 121 ARGs were dysregulated in the NF1$^{+/-}$ hippocampus (Fig. 4B). Of these ARGs, 71 were abnormally

downregulated and 50 upregulated in the $NF1^{+/-}$ hippocampus. This finding indicates that the LTP-related processes associated with these ARGs are disturbed in the $NF1^{+/-}$ hippocampus. The malfunction of these processes may contribute to the LTP impairments in the $NF1^{+/-}$ hippocampus (Costa et al., 2002). Among the LTP-related genes that are dysregulated in the $NF1^{+/-}$ hippocampus are αCaMKII and CREB1 (Table 2; Supplemental Table 1).

Lovastatin treatment of $NF1^{+/-}$ mice changes genomic expression patterns in the hippocampus.

A recent exciting study showed that treatment with lovastatin improved the learning performance of $NF1^{+/-}$ mice. Therefore, we sought to determine the effects of lovastatin treatment on the genomic expression in the $NF1^{+/-}$ hippocampus. We performed DNA microarray analysis to compare the hippocampal genomic expression of $NF1^{+/-}$ mice received 4 days of lovastatin treatment, which was effective to rescue memory deficits (Li et al., 2005), with that of control $NF1^{+/-}$ mice received sham (vehicle) treatment. We found the expression level of 2976 probe sets was altered by lovastatin treatment at $p<0.05$ and 682 genes at $p<0.01$. GO analysis indicated that the lovastatin-altered genes were involved in many biological processes, including cell-cell communication, cell signaling, transcription and cytoskeleton dynamics (Table 3). The results suggest that lovastatin affect genes in diverse biological processes that are disturbed in the $NF1^{+/-}$ hippocampus (Table 1). Of the NF1-disturbed genes ($p<0.05$), 377 changed their expression level after lovastatin treatment. We observed that lovastatin reversed the aberrant expression of some NF1-affected genes (Fig. 5A; Fig. 5B). For some other NF1-affected genes, lovastatin treatment caused further aberrant expression (Fig. 5C; Fig. 5D).

Next, we examined whether lovastatin affected activity-regulated genes (ARGs) that are regulated by LTP induction. We reasoned that lovastatin-induced memory improvement of $NF1^{+/-}$ mice may be accompanied by the reversal of the abnormal expression of some ARGs. To this end, we compared ARGs (Park et al., 2006) with the genes that are NF1-disturbed and lovastatin-regulated, and found that 210 NF1-disturbed ARGs were regulated by the lovastatin treatment ($p<0.05$) (Supplemental Table 3).

DISCUSSION

Neurofibromin negatively regulates MAPK signaling via Ras (Cichowski and Jacks, 2001). Therefore, NF1 mutations cause aberrant activation of MAPK signaling (Cichowski and Jacks, 2001). Previous studies have suggested an important role of MAPK signaling in regulating gene expression during synaptic plasticity and memory formation (English and Sweatt, 1997; Orban et al., 1999; Atkins et al., 2000; Davis et al., 2000; Thomas and Huganir, 2004). Hence, it is reasonable to hypothesize that abnormally expressed genes caused by NF1-mediated MAPK signaling activation underlie NF1-associated memory impairments. Consistent with this view, our studies indicate that NF1 mice are specifically impaired in memory consolidation but not encoding (acquisition) (Fig.1).

Functional assignment of NF1-affected hippocampal genes indicates that they are involved in a broad spectrum of molecular and cellular processes, including cell-cell communication, signal transduction, transcription and cytoskeleton dynamics (Table 1). Many genes in these biological pathways have been shown to be regulated during synaptic plasticity

and memory formation (Cavallaro et al., 2002; Levenson et al., 2004; Park et al., 2006). It is not clear at this stage whether the NF1 memory deficits are caused by the disturbance of one or multiple of these pathways. Nonetheless, the identified NF1-affected hippocampal genes provide a basis for further characterization of the specific processes that are causally relevant to the development of NF1 memory impairments.

We found that the expression of many genes encoding proteins involved in synaptic transmission is altered in the NF1 hippocampus (Table 2). These include proteins that are involved in regulation of synaptic vesicle recycling, such as Rabs, synaptotagmins and dynamins. An interesting feature is the bidirectional regulation of different members of the same protein family. For example, Rab1 and Rab2 are downregulated in the NF1 hippocampus, while Rab3A is upregulated; Synaptotagmin 1 is downregulated, while synaptotagmin7 is upregulated; Dynamin 2 is upregulated, while dynamin 3 is downregulated. It is intriguing to think that the bidirectional regulation of different members in the same protein family may provide a homeostatic mechanism to compensate the primary defects caused by NF1 mutation. In addition, the expression of glutamate receptors is also disturbed. For instance, NMDADR1 is downregulated, while AMPAR4 and mGluR5 are upregulated. Previous studies revealed a deficit of synaptic transmission in NF1 mice, which was considered to be caused by enhanced inhibition (Costa et al., 2002). Our findings reveal a disturbance of molecular processes underlying synaptic vesicle recycling; it would be interesting to determine whether these molecular disturbances contribute to the observed impairments of synaptic transmission.

Synaptic plasticity are thought to be accompanied by synapse remodeling (Engert and Bonhoeffer, 1999; Toni et al., 1999; Yuste and Bonhoeffer, 2001; Matsuzaki et al., 2004). We observed that some cytoskeleton regulatory genes are disturbed in the $NF1^{+/-}$ hippocampus

(Table 1). In addition, we found that, in the NF1$^{+/-}$ hippocampus, the expression of many genes encoding synaptic structural protein genes is altered. For example, integrinβ7, NCAM1 and transglutaminase2 are upregulated, whereas integrinβ6 and neurexin1 are downregulated (Table 2). The involvement of these classes of proteins in LTP has been demonstrated (Sanes and Lichtman, 1999; Park et al., 2006). Our findings suggest that synapse remodeling underlying synaptic plasticity may be dysregulated in the NF1$^{+/-}$ hippocampus.

The expression of long-term synaptic plasticity is controlled by synaptic signaling. The αCaMKII protein is one of the key signaling proteins that are enriched at synapses and critical for synaptic plasticity and memory formation (Lisman et al., 2002). Interestingly, in the NF1$^{+/-}$ hippocampus, αCaMKII gene is downregulated (Table 2). On the other hand, CaMKIIβ, which interacts with αCaMKII to form a holoenzyme, is upregulated (Table 2). Other signaling protein genes that are important for synaptic plasticity and disturbed in the NF1$^{+/-}$ hippocampus include CDK5 (Table 2) (Angelo et al., 2006). A major mechanism by which synaptic signaling regulates synaptic plasticity is to control gene transcription. CREB is a transcription factor that plays an important role in long-lasting synaptic plasticity and memory consolidation (Silva et al., 1998). Interestingly, CREB1 expression is decreased in the NF1$^{+/-}$ hippocampus (Table 1). The downregulation of CREB1 expression may contribute to the deficiency of memory consolidation in NF1$^{+/-}$ mice (Fig. 1).

Recent studies demonstrated that lovastatin suppresses MAPK activity and rescues memory deficits of NF1 mice(Li et al., 2005). We found that lovastatin treatment of NF1 mice for 1 week, which was sufficient to rescue memory deficits (Li et al., 2005), changed the expression of many genes in different functional groups (Table 3); some of them are synaptic plasticity-related genes. However, lovastatin-regulated genes are not significantly enriched with

NF1-affected genes. It is not clear at this stage whether lovastatin rescues NF1 memory deficits via reversing aberrant genomic expression.

MATERIALS AND METHODS

Nf1$^{+/-}$ mice breeding and genotyping

Nf1$^{+/-}$ mice (NCI) were bred with wild-type C57BL/6 mice and genotyped at 4 weeks using the REDExtract-N-Amp tissue PCR kit (Sigma –Aldrich) according to the manufacturer's instructions. PCR primers used were: NII - 5' GGT ATT GAA TTG AAG CAC 3', NIII - 5' ATT CGC CAA TGA CAA GAC 3', and 3'5' - 5' TTC AAT ACC TGC CCA AGG 3'. PCR reaction cycles were: 3 minutes at 94 ºC, a cycle of 1 minute at 94 ºC, 1 minute at 52 ºC, and 1 minute at 72 ºC repeated 35 times, and 10 minutes at 72 ºC.

Contextual discrimination

Eight week old mice were used for the contextual discrimination behavioral tests, which were performed as described (Frankland et al., 1998). All experiments were carried out blind with respect to genotypes. Two contextually different chambers, one for training with mild electric shocks (S) and another for control (C), were used in these behavioral tests. Chambers S and C were located in the same room and housed in separate sound-attenuated boxes. Three days prior to training, animals were handled daily for 5 minutes and allowed to freely explore both chambers S and C for 5 minutes (pre-exposure). On training day, in chamber S, animals were allowed to explore for 2.5 minutes before a 2 second 0.8 mA foot-shock was delivered. Mice were then observed for an additional 30 seconds before being returned to their home cages.

In chamber C, mice were allowed to explore for 3 minutes, without shocks, and then returned to their home cages. Contextual fear memory was measured by scoring freezing behaviors during the first 2.5 minutes of exploration immediately after the mouse was placed in a chamber. A mouse was scored freezing when it remains in a motionless posture, with only respiratory movement. Freezing was measured with a sampling method, in which two observers independently reviewed the video recording of an animal in each chamber and 2-second observations were taken every 5-seconds. If the animal remained motionless for the entire 2-second observation, then it was scored as freezing. Freezing data are presented as the percentage of time spent freezing during the training or testing period.

Lovastatin treatment

Lovastatin (mevinolin, Sigma-Aldrich) in the lactone form was dissolved in 55ºC ethanol and then NaOH was added (1M). The solution was left at room temperature for 30 minutes to complete the conversion of lovastatin to the sodium salt. The final lovastatin solution (4mg/ml) was adjusted to pH7.5 with HCl. Mice were injected with 10mg/kg of lovastatin or the vehicle control subcutaneously once per day for 4 days. On the 4th day, 6 hours following the final injection, mice were sacrificed to collect the hippocampus for RNA extraction.

Tissue collection / RNA extraction

Whole hippocampi were dissected and collected, with one hippocampus immediately used for RNA extraction and the other flash-frozen and stored at -80 ºC for additional RNA extraction or other assays. Total RNA was extracted from fresh, whole hippocampus using the RNeasy kit (Qiagen). The hippocampus was first homogenized in lysis buffer (supplied with the

RNeasy kit) for 30 seconds using a mortar and pestle. The lysate was then centrifuged through a QIAshredder column (Qiagen) to further homogenize the tissue. RNA was extracted using the RNeasy kit following the manufacturer's directions. The extracted total RNA was flash-frozen and stored at -80 ºC.

Microarray analysis

Microarray processing and hybridization was performed by the University of California, Irvine DNA Core Facility. Prior to processing for microarray hybridization, the quality of each RNA sample was verified with the Agilent 2100 Bioanalyzer. Total RNA was reverse transcribed into cDNA, converted to biotin-labeled cRNA, and then hybridized onto individual Affymetrix Mouse Genome 430 2.0 Array GeneChips (MG430v2) arrays following the manufacturer's instructions.

Two sets of normalized gene expression values were obtained using GCOS 1.4 (Affymetrix) and dCHIP. While the GCOS algorithm is suitable for determining high-level expression values, for low-level expression values, the Model-based expression index (MBEI) analysis performed in dChip improves the accuracy of the expression values by reducing the variability of low expressing targets. Pair-wise statistical comparisons of microarray data from *Nf1*$^{+/-}$ mice with WT control mice was performed using CyberT, which performs t-tests that incorporate a Bayesian estimate of the variance of the microarray expression data to compensate for a low number of experimental replicates (http://visitor.ics.uci.edu/genex/cybert/). Genes that were differentially expressed between *Nf1*$^{+/-}$ and WT control mice at statistically significant levels ($p<0.05$) were identified and compiled in list for further analysis. Hierarchical clustering

of expression profiles of *Nf1*$^{+/-}$ and WT control mice was performed using dChip and GeneSpring (Silicon Genetics).

Real-time quantitative RT-PCR verification

Real time quantitative RT-PCR was performed with an ABI Prism 7900HT sequence detection system and LUX fluorogenic primers (Invitrogen). For each target gene, fluorophore-labeled LUX forward primers and their corresponding unlabeled reverse primers were designed using LUX Designer (http://www.invitrogen.com/lux). Real time quantitative RT-PCR was performed using 0.1 μg of total RNA (from the same samples used for the microarray analysis) and the SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen) according to the manufacturer's instructions. PCR reaction cycles were: 15 minutes at 50 ºC, 2 minutes at 95 ºC, and then 50 cycles of 15 seconds at 95 ºC and 30 seconds at 60 ºC. The relative difference of expression levels between *Nf1*$^{+/-}$ and WT control mice for each target gene was calculated by a relative cycle threshold method ($2^{-\Delta\Delta C_T}$) using PGK1 as a reference.

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FIGURE LEGENDS

Figure 1. NF1$^{+/-}$ mice display learning deficits in a contextual discrimination task. (A). At the training day, age- and gender-matched wild-type and NF1$^{+/-}$ mice were placed in the training (S) or control (C) chambers to explore for 2.5 min. Mild electric shocks were then delivered in chamber S but not in chamber C. Freezing behaviors during the period before shock delivery were scored. Wild-type and NF1$^{+/-}$ mice were not different in their freezing behaviors in both chambers. (B). 1-day memory tests. One day after training, mice were exposed to chamber S or C. In chamber S, compared with the training day (A), both wild-type and NF1$^{+/-}$ mice expressed 4-fold more freezing behaviors, suggesting the formation of fear memories about chamber S. Mice of both genotypes showed similar level of freezing in chamber C, similar to that from the training day (A). (C). 7-day memory tests after one session of training. Mice trained in (A) were tested 7 days after. Note that wild-type mice showed a significantly higher level of freezing than NF1$^{+/-}$ mice in chamber S. (D). 7-day memory tests after two sessions of training. Mice received two sessions of training on the first and second days and memory tests were performed on 7th day. Mice of both genotypes displayed similar levels of freezing in S chamber. Ten to fifteen animals were included in each group.

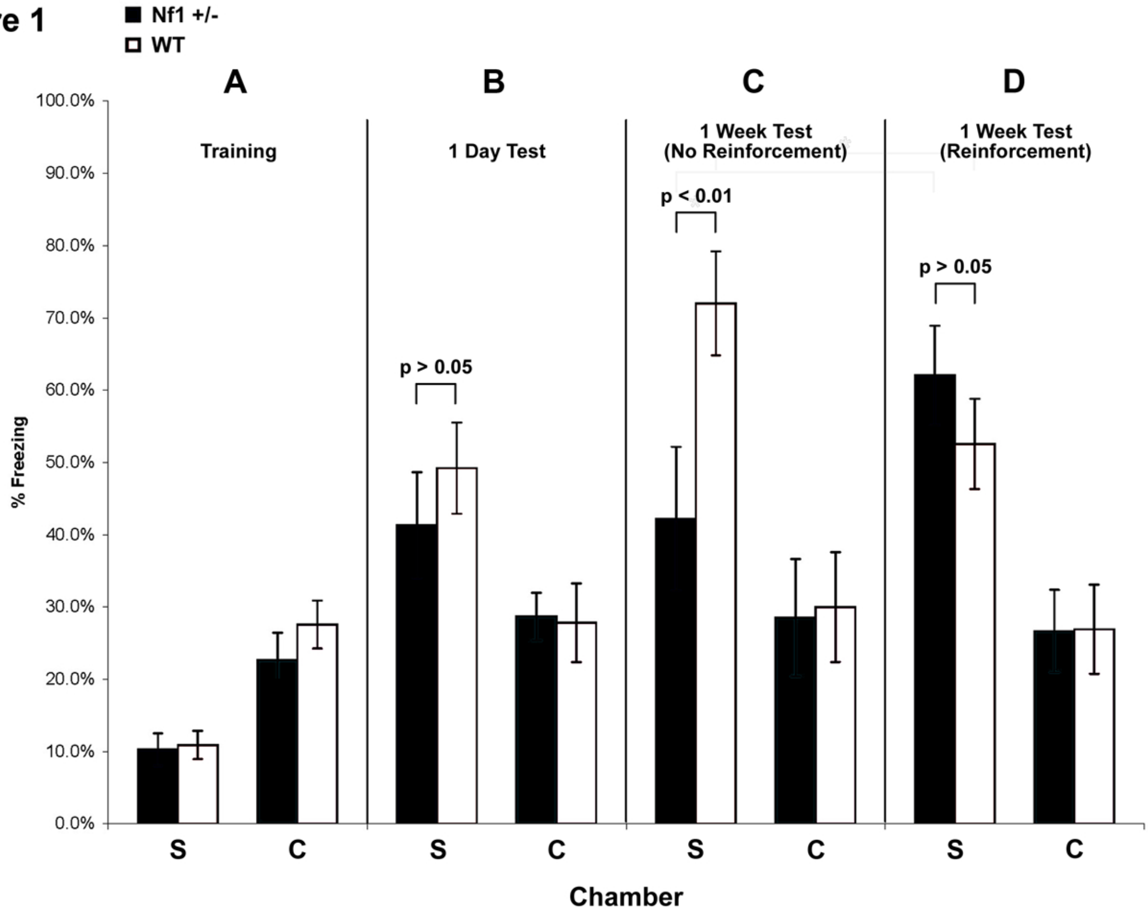
Figure 2. A scatter plot of expression values of all genes on microarrays. Genes that were differentially expressed in the wild-type and NF1$^{+/-}$ hippocampus are indicated by dots outside the diagonally centered channel.

Figure 3. Real-time RT-PCR confirmation of altered expression of a set of genes in the $NF1^{+/-}$ hippocampus.

Figure 4. Aberrant expression of synapse- and plasticity-related genes in the $NF1^{+/-}$ hippocampus. (A). Abnormally down- (left) or up-regulated (right) synapse-related genes. (B) Abnormally down- or up-regulated ARGs.

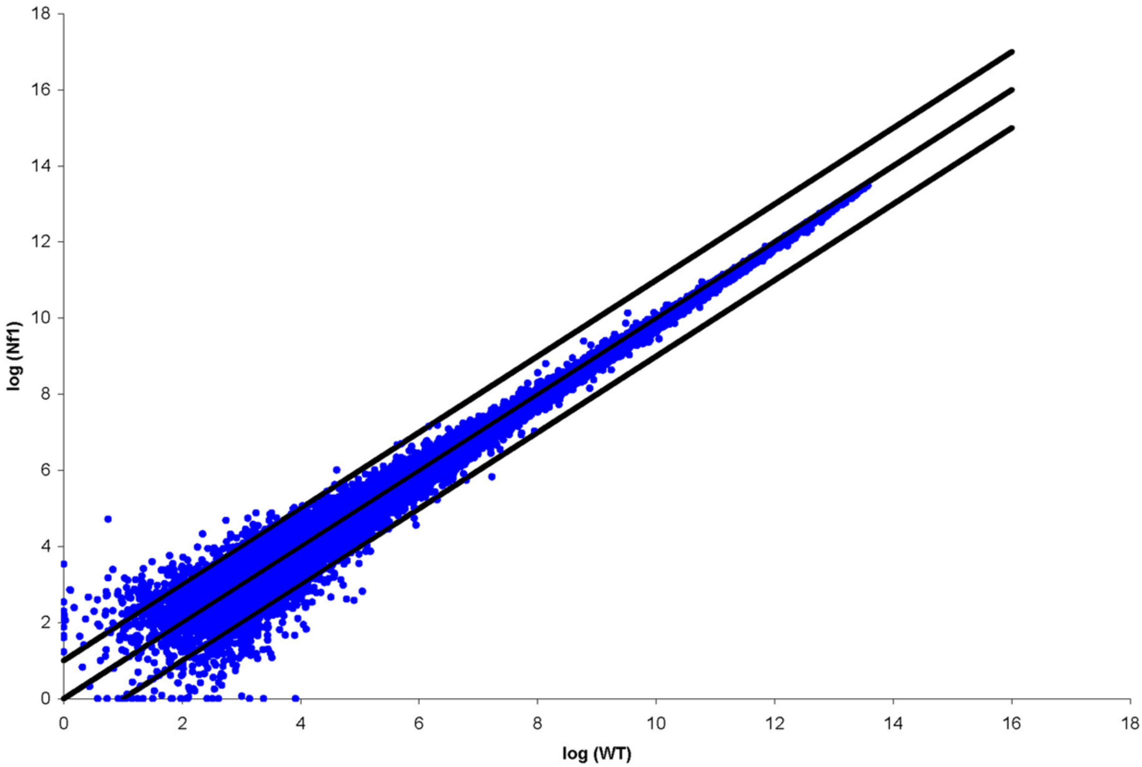
Figure 5. Effects of lovastatin on hippocampal genomic expression of $NF1^{+/-}$ mice. (A) A group of genes that were down-regulated genes in the $NF1^{+/-}$ hippocampus were up-regulated by lovastatin. (B). A group of genes that were up-regulated genes in the $NF1^{+/-}$ hippocampus were down-regulated by lovastatin. (C). A group of genes that were up-regulated genes in the $NF1^{+/-}$ hippocampus were also up-regulated by lovastatin. (D) A group of genes that were down-regulated genes in the $NF1^{+/-}$ hippocampus were also down-regulated by lovastatin.

Figure 1



Nf1 +/-
WT
A
B
C
D
Training
1 Day Test
1 Week Test
(No Reinforcement)
1 Week Test
(Reinforcement)
p > 0.05
p < 0.01
p > 0.05
% Freezing
100.0%
90.0%
80.0%
70.0%
60.0%
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
S
C
S
C
S
C
S
C
Chamber

Figure 2



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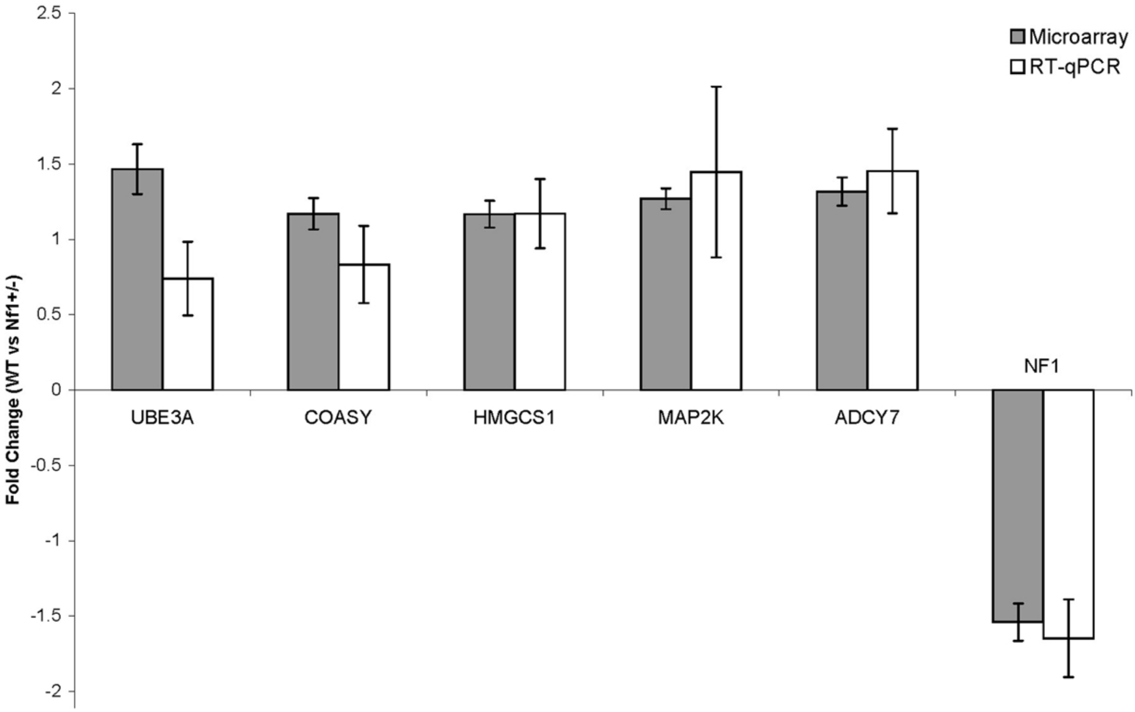
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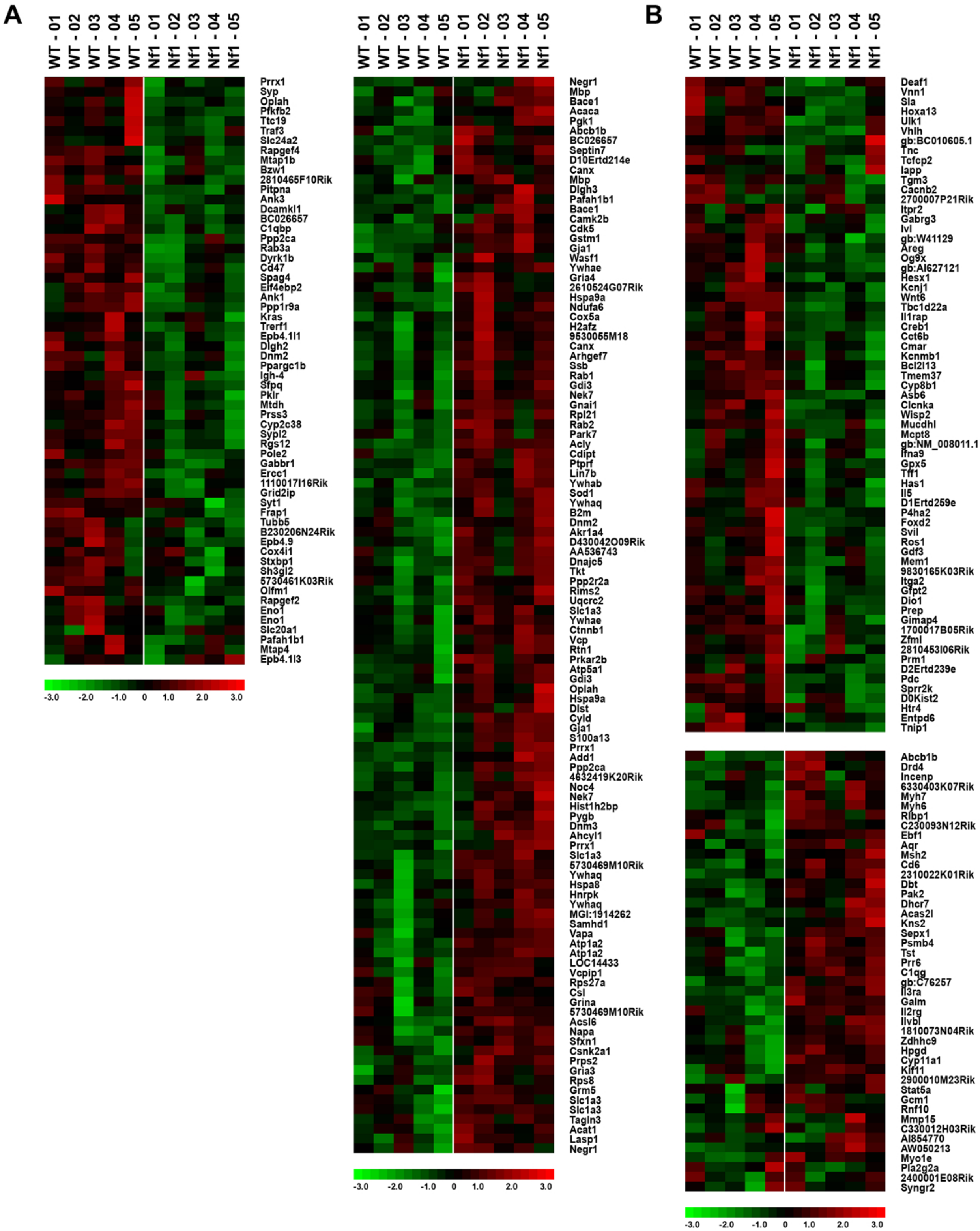
COASY
HMGCS1
MAP2K
ADCY7
NF1

Figure 4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WT - 02
WT - 03
WT - 04
WT - 05
Nf1 - 01
Nf1 - 02
Nf1 - 03
Nf1 - 04
Nf1 - 05
Prrx1
Syp
Oplah
Pfkfb2
Ttc19
Traf3
Slc24a2
Rapgef4
Mtap1b
Bzw1
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Nf1 - 02
Nf1 - 03
Nf1 - 04
Nf1 - 05
Negr1
Mbp
Bace1
Acaca
Pgk1
Abcb1b
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Septin7
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Canx
Mbp
Dlgh3
Pafah1b1
Bace1
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Cdk5
Gstm1
Gja1
Wasf1
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Gnai1
Rpl21
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Acly
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Sod1
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Vcp
Rtn1
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Negr1
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1.0
2.0
3.0
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WT - 02
WT - 03
WT - 04
WT - 05
Nf1 - 01
Nf1 - 02
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Nf1 - 04
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Sla
Hoxa13
Ulk1
Vhlh
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Tgm3
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Clcnka
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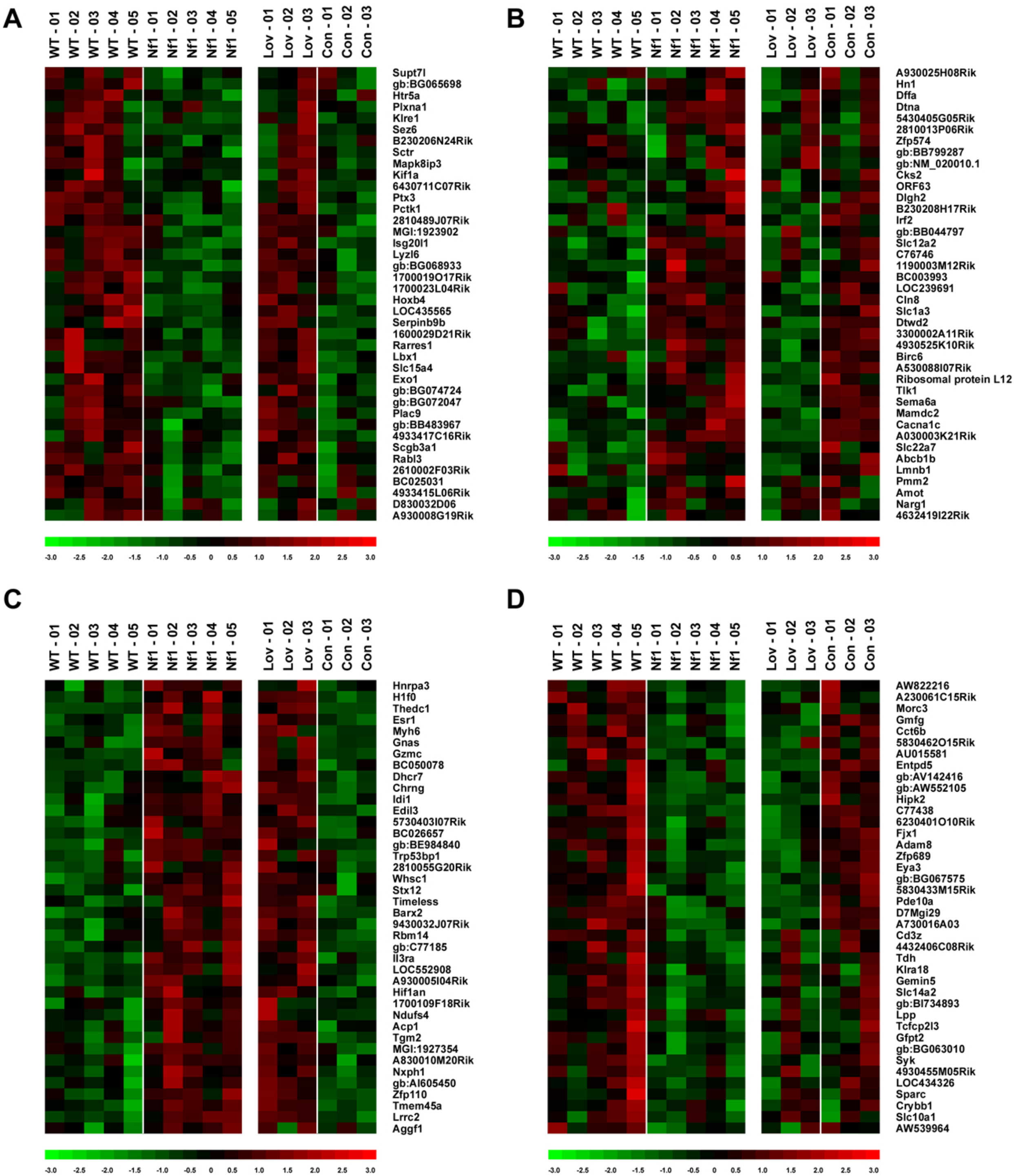
Figure 5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Lov - 01
Lov - 02
Lov - 03
Con - 01
Con - 02
Con - 03
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WT - 02
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WT - 04
WT - 05
Nf1 - 01
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2.5
3.0

Table 1: Ontology groups of differentially expressed genes in the $Nf1^{+/-}$ hippocampus ($p<0.01$)

	Probe Set	Gene Symbol	Gene Description	Up or Down Regulated
Cell-cell Communicaiton (99)	1415800_at	Gja1	gap junction membrane channel protein alpha 1	UP
	1420744_at	Chrnb2	cholinergic receptor, nicotinic, beta polypeptide 2 (neuronal)	DOWN
	1421531_at	Akap3	A kinase (PRKA) anchor protein 3	UP
	1449206_at	Sypl2	synaptophysin-like 2	DOWN
	1450043_at	Fzd7	frizzled homolog 7 (Drosophila)	DOWN
Signal Transduction (82)	1417605_s_at	Camk1	calcium/calmodulin-dependent protein kinase I	UP
	1418098_at	Adcy4	adenylate cyclase 4	UP
	1426233_at	Map2k4	mitogen activated protein kinase kinase 4	DOWN
	1428813_a_at	Drd1ip	dopamine receptor D1 interacting protein	DOWN
	1435196_at	Ntrk2	neurotrophic tyrosine kinase, receptor, type 2	UP
Cellular Transport (74)	1446431_at	Dnm3	Dynamin 3	DOWN
	1421990_at	Syt1	synaptotagmin I	DOWN
	1426578_s_at	Snapap	SNAP-associated protein	UP
	1432004_a_at	Dnm2	dynamin 2	UP
	1446826_at	Xpo7	exportin 7	DOWN
Transcription (71)	1416149_at	Olig1	oligodendrocyte transcription factor 1	UP
	1418091_at	Tcfcp2l1	transcription factor CP2-like 1	UP
	1420811_a_at	Ctnnb1	catenin (cadherin associated protein), beta 1, 88kDa	UP
	1426437_s_at	Hdac3	histone deacetylase 3	DOWN
	1452529_a_at	Creb1	cAMP responsive element binding protein 1	DOWN
Protein Modification (59)	1421340_at	Map3k5	mitogen activated protein kinase kinase kinase 5	DOWN
	1424116_x_at	Ppp5c	protein phosphatase 5, catalytic subunit	DOWN
	1430022_at	Uble1a	ubiquitin-like 1 (sentrin) activating enzyme E1A	DOWN
	1431825_at	Stk23	serine/threonine kinase 23	DOWN
	1435757_a_at	Uqcrc2	ubiquinol cytochrome c reductase core protein 2	UP
Cytoskeleton (17)	1422481_at	Krt2-1	keratin complex 2, basic, gene 1	DOWN
	1448554_s_at	Myh6	myosin, heavy polypeptide 6, cardiac muscle, alpha	UP
	1450112_a_at	Gas2	growth arrest specific 2	UP
	1453193_s_at	Kif12	kinesin family member 12	DOWN
	1456618_at	Mark4	MAP/microtubule affinity-regulating kinase 4	DOWN
Cell Differentiation (19)	1416714_at	Irf8	interferon regulatory factor 8	UP
	1417419_at	Ccnd1	cyclin D1	UP
	1422300_at	Nog	noggin	UP
	1438067_at	Nf1	neurofibromatosis 1	DOWN
	1446185_at	Frap1	FK506 binding protein 12-rapamycin associated protein 1	DOWN

Notes: Representative examples given for each group. Total number of genes given in ( ).

Table 2. Differentially expressed synapse-related genes in the Nf1$^{+/-}$ hippocampus ($p<0.05$)

	Probe Set	Gene Symbol	Gene Description	Up or Down Regulated
Vesicle recycling	1416465_a_at	Vapa	vesicle-associated membrane protein, associated protein A	DOWN
	1418621_at	Rab2	RAB2, member RAS oncogene family	DOWN
	1420506_a_at	Stxbp1	syntaxin binding protein 1	DOWN
	1421990_at	Syt1	synaptotagmin I	DOWN
	1422589_at	Rab3a	RAB3A, member RAS oncogene family	UP
	1422809_at	Rims2	regulating synaptic membrane exocytosis 2	UP
	1443150_at	Dnm2	Dynamin 2	UP
	1446431_at	Dnm3	Dynamin 3	DOWN
	1446641_at	Syt7	synaptotagmin 7	UP
	1448210_at	Rab1	RAB1, member RAS oncogene family	DOWN
	1449206_at	Sypl2	synaptophysin-like 2	DOWN
	1456249_x_at	Syp	Synaptophysin	DOWN
Synaptic receptor	1420563_at	Gria3	glutamate receptor, ionotropic, AMPA3 (alpha 3)	DOWN
	1425595_at	Gabbr1	gamma-aminobutyric acid (GABA-B) receptor, 1	DOWN
	1436297_a_at	Grina	glutamate receptor, ionotropic, N-methyl D-asparate-associated protein 1	DOWN
	1436772_at	Gria4	Glutamate receptor, ionotropic, AMPA4 (alpha 4)	UP
	1450202_at	Grin1	glutamate receptor, ionotropic, NMDA1 (zeta 1)	DOWN
	1450310_at	Grid2ip	glutamate receptor, ionotropic, delta 2 (Grid2) interacting protein 1	DOWN
	1455272_at	Grm5	glutamate receptor, metabotropic 5	UP
Synaptic signaling	1419034_at	Csnk2a1	casein kinase II, alpha 1 polypeptide	DOWN
	1420842_at	Ptprf	protein tyrosine phosphatase, receptor type, F	DOWN
	1422590_at	Cdk5	cyclin-dependent kinase 5	DOWN
	1424482_at	Arhgef7	Rho guanine nucleotide exchange factor (GEF7)	DOWN
	1441734_at	Camk2a	Calcium/calmodulin-dependent protein kinase II, alpha	DOWN
	1444875_at	Ppp2ca	Protein phosphatase 2a, catalytic subunit, alpha isoform	UP
	1446651_at	Rapgef2	Rap guanine nucleotide exchange factor (GEF) 2	DOWN
	1455869_at	Camk2b	Calcium/calmodulin-dependent protein kinase II, beta (Camk2b)	UP
	1456475_s_at	Prkar2b	protein kinase, cAMP dependent regulatory, type II beta	DOWN
Synaptic structure	1418741_at	Itgb7	integrin beta 7	UP
	1433428_x_at	Tgm2	transglutaminase 2, C polypeptide	UP
	1420811_a_at	Ctnnb1	catenin (cadherin associated protein), beta 1	DOWN
	1421850_at	Mtap1b	microtubule-associated protein 1 B	DOWN
	1423846_x_at	Tuba2	tubulin, alpha 2	DOWN
	1432281_a_at	Itgb6	integrin beta 6	DOWN
	1433413_at	Nrxn1	neurexin I	DOWN
	1443291_at	Ank1	Ankyrin 1, erythroid (Ank1), mRNA	UP
	1443702_at	Mtap4	microtubule-associated protein 4	DOWN
	1448346_at	Cfl1	cofilin 1, non-muscle	DOWN
	1450438_at	Ncam1	neural cell adhesion molecule 1	UP
	1452124_at	Ank3	ankyrin 3, epithelial	DOWN
	1454651_x_at	Mbp	myelin basic protein	DOWN

Notes: Representative examples given for each group.

Table 3: Ontology groups of Lovastatin-affected genes in the Nf1+/- hippocampus (p<0.01)

	Probe Set	Gene Symbol	Gene Description	Up or Down Regulated by Lovastatin
Cell-cell Communicaiton (46)	1419245_at	Rab14	RAB14, member RAS oncogene family	UP
	1425549_at	Psen1	presenilin 1	UP
	1431050_at	Rps6ka5	ribosomal protein S6 kinase, polypeptide 5	UP
	1451711_at	Wnt9b	wingless-type MMTV integration site 9B	DOWN
	1459256_at	Nrp2	Neuropilin 2	UP
Transcription (37)	1417516_at	Ddit3	DNA-damage inducible transcript 3	UP
	1420410_at	Nr5a2	nuclear receptor subfamily 5, group A, member 2	UP
	1425630_at	Sin3b	transcriptional regulator, SIN3B (yeast)	UP
	1449566_at	Nkx2-5	NK2 transcription factor related, locus 5 (Drosophila)	UP
	1450482_a_at	Pitx2	paired-like homeodomain transcription factor 2	DOWN
Signal Transduction (37)	1421465_at	Wnt2b	wingless related MMTV integration site 2b	DOWN
	1436251_at	Pde1c	phosphodiesterase 1C	UP
	1450176_at	Ern1	endoplasmic reticulum (ER) to nucleus signalling 1	UP
	1451714_a_at	Map2k3	mitogen activated protein kinase kinase 3	UP
	1459912_at	Map4k4	mitogen-activated protein kinase kinase kinase kinase 4	UP
Cellular Transport (19)	1422809_at	Rims2	regulating synaptic membrane exocytosis 2	UP
	1452747_at	Atp13a2	ATPase type 13A2	UP
	1454077_at	Vti1a	vesicle transport, interaction with t-SNAREs homolog 1A	DOWN
	1460129_at	Slc6a2	solute carrier family 6, member 2	UP
	1460712_s_at	Ap1g1	adaptor protein complex AP-1, gamma 1 subunit	UP
Protein modification (19)	1419988_at	Map3k7	mitogen activated protein kinase kinase kinase 7	UP
	1426004_a_at	Tgm2	transglutaminase 2, C polypeptide	UP
	1447617_at	Cdk2	cyclin-dependent kinase 2	DOWN
	1449013_at	Eef2k	eukaryotic elongation factor-2 kinase	UP
	1456992_at	Ube3c	ubiquitin protein ligase E3C	UP
Cell Differentiation (10)	1418054_at	Neurod4	neurogenic differentiation 4	UP
	1418420_at	Myod1	myogenic differentiation 1	UP
	1425264_s_at	Mbp	myelin basic protein	UP
	1425703_at	Ppard	peroxisome proliferator activator receptor delta	DOWN
	1449773_s_at	Gadd45b	growth arrest and DNA-damage-inducible 45 beta	UP
Cytoskeleton (9)	1420248_at	Tubg2	tubulin, gamma 2	UP
	1420358_at	Krtap13	keratin associated protein 13	UP
	1421053_at	Kif1a	kinesin family member 1A	UP
	1435046_at	Myo1d	Myosin ID (Myo1d), mRNA	UP
	1453997_a_at	Nes	nestin	UP

Notes: Representative examples given for each group. Total number of genes given in ( ).